

Influence of protein-phenolic complex on the antioxidant capacity of flaxseed (*Linum usitatissimum* L.) products

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Abstract

The impact of the naturally-present phenolic compounds and/or proteins on the antioxidant capacity of flaxseed products- phenolic fraction, protein concentrates and hydrolysates- before and after simulated gastrointestinal digestion was studied. For that, whole and phenolic reduced products were assessed. Four glycosylated phenolic compounds – secoisolariciresinol and ferulic, p-coumaric and caffeic acids – were identified in flaxseed products. Phenolic fraction exerts the highest antioxidant capacity that increased by alkaline hydrolysis and by simulated gastrointestinal digestion. The action of Alcalase[®] and digestive enzymes resulted in an increase of the antioxidant capacity of whole and phenolic reduced products. Principal component analysis showed that proteinaceous samples act as antioxidant is by H⁺ transfer, while those samples containing phenolic compounds exert their effects by both electron donation and H⁺ transfer mechanisms. Protein/peptide-phenolic complexation, confirmed by fluorescence spectra, exerted a positive effect on the antioxidant capacity, mainly in protein concentrates.

Keywords

Flaxseed, antioxidant capacity, protein, phenolic compounds, enzymatic hydrolysis, simulated gastrointestinal digestion

Introduction

Several studies have shown the antioxidant potential of peptides released from enzymatic hydrolysis of different protein sources.¹⁻³ This source of antioxidants has attractive characteristics for the food industry, once it is non-toxic and has recognized nutritional value.¹ In food industry, peptides can be an alternative to the use of synthetic antioxidants, preventing lipid peroxidation and maintaining the sensory characteristics of the products.¹ In the human body, they may assist the antioxidant defense system in the prevention or deceleration of the progression of oxidative stress-associated diseases.^{4,5}

It has been reported that procedures used to obtain protein plant protein isolates can also facilitate the extraction of polyphenols.^{6,7} After enzymatic hydrolysis, both released peptides and phenolic compounds might be responsible for the antioxidant activity of hydrolysates.^{1,8} In this respect, the antioxidant activity of plant-derived matrices has been associated with protein components, phenolic compounds and their complexes, although few studies on protein hydrolysates have considered the presence and contribution of phenolic compounds.⁹

Flaxseed is an oilseed widely studied for its beneficial health effects. It is a source of alpha linolenic fatty acids, phenolic compounds and soluble fiber, has anti-inflammatory and antioxidant capacities, and has been related to reduce risk of chronic diseases such as cancer, obesity, and diabetes.^{10,11} Flaxseed is the richest source of plant lignans, due to its high content of secoisolariciresinol diglucoside (SDG).¹² In this seed, SDG along with non-lignan phenolic compounds, such as ferulic, p-coumaric and caffeic acids, are constituents of an oligomeric structure called lignan macromolecule.^{13,14} These phenolic compounds have phytoestrogenic and antioxidant properties^{12,14} and, therefore, they may exert potential benefits on human health.¹⁵

A previous study carried out in our laboratory with flaxseed protein concentrates containing phenolic compounds have shown that simulated gastrointestinal digestion was equal or more effective than Alcalase[®] hydrolysis to obtain antioxidant hydrolysates.¹⁶ However, the specific contribution of phenolic compounds and peptides on the antioxidant capacity of flaxseed protein hydrolysates, as well as on other plant-derived hydrolysates, is not fully elucidated. Therefore, the aim of the present study was to evaluate the influence of naturally-present phenolic compounds and their complexes with proteins on the antioxidant potential of flaxseed products before and after simulated gastrointestinal digestion.

MATERIALS AND METHODS

Reactives

Partially defatted brown flaxseed meal (FM) was obtained from Cisbra Ltd. (Panambi, RS, Brazil). Alcalase[®] 2.4 L, pepsin, pancreatin, bile salts, Folin-Ciocalteu reagent, gallic acid, [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] (Trolox), 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), sodium fluorescein, secoisolariciresinol, caffeic, *p*-coumaric and ferulic acids were purchased from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA) and acetonitrile were purchased from Merck (Hohenbrunn, Germany). All other chemicals and reagents were of analytical grade.

Preparation of flaxseed defatted flour, protein concentrates and phenolic fractions

A flow chart of the preparation of flaxseed protein products and phenolic fraction is shown in Figure 1.

To obtain the defatted flaxseed meal (DFM), FM was defatted with hexane in a ratio of 1:3 (w/v) for 24 h at room temperature. For polyphenols extraction, three consecutive steps

with 63% ethanol solution (v/v) were performed.¹³ Two first steps were performed for 4 h under stirring and room temperature, whereas the last one was made overnight. After each extraction phase, DFM was centrifuged at 2500 x g for 30 min, and filtered obtaining the phenolic-reduced DFM (phr-DFM) and the phenolic isolate (Phi).

Flaxseed protein concentrate (FPC) and phenolic-reduced FPC (phr-FPC) were prepared from DFM and phr-DFM, respectively, following Dev & Quensel protocol.¹⁷ The corresponding source sample was dispersed in deionized water at a product:water ratio of 1:10 (w/w), and after adjusting its pH to 9.0 with 0.5 M NaOH, the solution was stirred at room temperature for 30 min and centrifuged (2500 x g/30 min; 25 °C). The supernatant containing protein was filtered and its pH adjusted to 4.2 with 0.5 M HCl. The precipitated protein was then separated by centrifugation (2500 x g/30 min), washed three times with acidified water (pH 4.2), and suspended in deionized water adjusting its pH to 6.0 with 0.5 M NaOH. Flaxseed products were freeze-dried and stored at -20 °C until their use.

Hydrolysis of flaxseed products

The hydrolysis of FPC and phr-FPC was performed with Alcalase[®] under the following conditions: protein concentration of 5% (w/v), 60 °C, pH 8.5, and enzyme substrate (E:S) ratio 1:90 (w/w). The hydrolysis reaction was monitored using the pH-stat method using an automatic titrator DL model Metler 21 (Schwerzenbach, Switzerland) with a stirring system coupled to a thermostatic bath. After 180 min, the reaction was stopped by heating at 90 °C for 10 min. Then, the pH of the hydrolysates was adjusted to 6.0, and they were freeze-dried, and stored at -20 °C. The degree of hydrolysis (% DH) of flaxseed protein hydrolysate (FPH) and phenolic reduced flaxseed protein hydrolysate (phr-FPH) obtained from FPC and phr-FPC, respectively, was calculated according to the equation described by Adler Nissen.¹⁸

To assess whether the conditions for obtaining the protein hydrolysate may change the phenolic compounds present in the FPC, Phi (5% w/v) was subjected to the same conditions of temperature and time of the hydrolysis reaction, but without addition of Alcalase[®] to obtain the phenolics hydrolysate (Phh) (Figure 1).

Chemical composition

The chemical composition of flaxseed products was determined according to AOAC procedures.¹⁹ Protein and lipid contents were determined according to Kjeldahl (N x 6.25)¹⁹, and Bligh & Dyer²⁰ methods, respectively.

Characterisation of flaxseed products by RP-HPLC

The chromatographic analysis of flaxseed products was carried out using a reverse-phase high-performance liquid chromatography (RP-HPLC) system with an automatic injector and a diode-array absorbance detector (Agilent, 1200 Series, Snoqualmie, WA, USA). Separation was carried out onto a Luna C18 column (250 mm x 4.6 mm, Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL/min. The mobile phase was constituted by solvent A (0.04% TFA in water) and solvent B (0.03% TFA in acetonitrile). The gradient was from 0 to 80% of solvent B over 40 min. The absorbance was measured at 214 and 280 nm. The samples were filtered through a 45-µm membrane, and 50 µL were injected. Sample concentration of flaxseed flours, concentrates and hydrolysates was adjusted to 8 mg/mL. In the case of digested samples, concentration was adjusted to 4 mg/mL. The Star Chromatography Workstation software (Agilent) was used to record and process data.

Identification of phenolic compounds by UPLC-MS/MS

The identification of ferulic, *p*-coumaric and caffeic acids and SDG was carried out using a UPLC-Q-ToF system comprised of an AcquityTM UPLC system coupled to a

XevoTM G2-XS Q-Tof (Waters Corp., Milford, MA, USA), with an electrospray source ionization (ESI) in negative mode. The instrument control and data processing were performed by MassLynx software (Waters Corp.) version 4.1. Samples were analyzed in MSE mode, in which precursor and fragment information are collected from the same analysis. For the MS operating conditions the following parameters were set: capillary voltage 2.5 kV, cone voltage 30 V, source temperature 150 °C, desolvation temperature 550 °C, cone gas flow 50 L/h and desolvation gas flow 900 L/h. The instrument was previously calibrated with a sodium formate solution, and all runs were acquired with real time lockspray correction for mass accuracy (deprotonated rutin ion, mass/charge (m/z) 609.1456). Spectra were acquired every 0.1 s, on a m/z range of 100-1200. High energy spectra were acquired from m/z 50-1200 using a collision energy ramp from 20-30 eV.

The chromatographic separation was carried out on a BEH C18 column (50 mm \times 2.1 mm \times 1.7 μ m) (Waters Corp.). Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient program was as follows: B was ramped from 5-95% in 4 min, followed by a 0.5 min lapse at 95% B. Then, mobile phase composition was restored to initial conditions for 0.5 min. Flow rate was set to 0.6 mL/min, the injection volume was 5 μ L, and the column oven and sample manager were kept at 45 °C and 10 °C respectively. Each standard or sample was properly diluted in ultrapure water, and filtered through 0.45 μ m polytetrafluoroethylene membrane before being injected onto the system.

Analysis of protein–phenolic complex by fluorescence spectroscopy

The intrinsic fluorescence analysis was performed to study the formation of the protein-phenolic complex among proteins/peptides and polyphenols according to Kanakis, et al.²¹ with some modifications. FPC, phr-FPC, FPH and phr-FPH were dispersed in aqueous solutions at a protein concentration of 4 mg/mL. The fluorescence spectra of the Phi and Phh

(4 mg sample/mL) were also recorded. All solutions were prepared at 24.0 ± 1 °C and kept in the dark. Fluorescence spectra were recorded at $\lambda_{\text{exc}} = 280$ nm and λ_{emi} from 290 to 500 nm, and data were acquired using an ISS PC1 Fluorimeter (Champaign, IL, USA).

Simulated gastrointestinal digestion

Simulated gastrointestinal digestion was performed as reported by Martos et al.²² with modifications. The samples were dispersed in gastric juice (35 mM NaCl), and the pH was adjusted to 2.0 with 1 M HCl, thus obtaining a concentration of 5.9 mg protein/mL (DFM, phr-DFM, FPC, phr-FPC, FPH, phr-FPH) and 1.2 mg phenolic compounds/mL (Phi and Phh). Mixtures were left in a water bath at 3 °C for 15 min under constant stirring. Then, pepsin was added (E:S 1:20, w/w), and the pH was again adjusted to 2.0. The mixture was left in a thermostatic bath at 37 °C for 60 min under stirring. At the end of gastric phase, the pH of samples was adjusted to 6.8 with 1 M NaHCO₃, 1 M CaCl₂, and 9 mg/mL bile salt and they were maintained in a water bath at 37 °C for 15 min under stirring. Then, pancreatin was added (E:S 1:10, w/w), the pH was adjusted to 6.8 with 1 M NaHCO₃, and the volume was made up to 4 mL with deionized water. The intestinal digestion was carried out at 37 °C for 60 minutes under stirring. To stop the reaction, the digest was heated at 90 °C for 10 min under stirring and centrifuged (11000 x g) for 15 min. The supernatants (digests) were frozen and kept at -20 °C until further use.

Antioxidant capacity

The antioxidant capacity of the samples before and after simulated digestion was measured in the aqueous extracts. To obtain the extracts, lyophilized non digested samples (1% w/v) and digested samples (3% v/v) were suspended in deionized water, shaken for 30

min, centrifuged at 36000 x g for 30 min at 10°C, filtered through N°1 Whatman qualitative filter paper, and stored at -20 °C in dark until use. All the antioxidant capacity assays were carried out using a Synergy™ HT Multi-Mode Microplate Reader (Biotek®, Winooski, VT, USA).

Folin-Ciocalteu reagent reducing substances (FCRRS). The procedure was carried out according to Medina.²³ Briefly, 450 µL of deionized water and 50 µL of appropriately diluted samples, gallic acid standard solutions (0, 50, 100, 200, 300, 400, 500, 600 µg/mL) or deionized water for blank were added and mixed. The Folin-Ciocalteu reagent (50 µL) was added, mixed, and allowed to react for 5 min. Then, 500 µL of 7% Na₂CO₃ and 200 µL of deionized water were added and mixed. The mixture was left to react at room temperature in the dark for 90 min. The absorbance was measured at 760 nm and the results were expressed as mg gallic acid equivalent (GAE) per gram of sample (mg GAE/g sample).

Ferric reducing antioxidant power (FRAP). The FRAP assay was carried out according to Benzie & Strain²⁴ with modifications. In the dark, 30 µL of sample extract, standard or blank was mixed with 90 µL of water and 900 µL of the FRAP reagent (450 µL of 0.3 M acetate buffer, pH 3.6, 225 µL of 10 mmol TPTZ in 40 mmol HCl and 225 µL of 20 mmol FeCl₃). The mixture was incubated at 30 °C for 30 min. The absorbance was measured at 595 nm and the results were expressed as µmol of Trolox equivalent (TE) per gram of sample (µmol TE/g sample).

Oxygen radical absorbance capacity (ORAC). ORAC assay was performed according to Davalos et al.²⁵ Briefly, 20 µL of sample extract and 120 µL of sodium fluorescein in potassium phosphate buffer (pH 7.4) (final concentration 0.378 µg/mL) were mixed in water with 60 µL of AAPH (final concentration 108 mg/mL). Potassium phosphate buffer was used as a blank. Trolox solutions (25-500 mM) were used as standard. Fluorescence was measured every minute for 80 min with an excitation wavelength of 485 nm

and an emission wavelength of 520 nm. The antioxidant capacity was expressed as $\mu\text{mol TE/g}$ sample, based on the area under the curve (AUC) for the decline in the fluorescence time.

Metal chelation activity. Fe^{2+} -chelating activity was determined by measuring the formation of the Fe^{2+} -ferrozine complex according to Carter ²⁶ with adaptations. Samples were diluted (0.4-6.4 mg/mL) in 100 mM sodium acetate buffer (pH 4.9), stirred for 30 min and centrifuged at $27821 \times g$. Sample solution (250 μL) was mixed with 30 μL FeCl_2 (50 $\mu\text{g/mL}$), and incubated for 30 min at room temperature. Then, ferrozine (12.5 μL , 40 mM) was added. Ethylenediamine tetraacetic acid (EDTA) was used as a positive control at the same concentration used for samples. The chromophore formed by binding of Fe^{2+} ions to ferrozine was measured at 562 nm. Iron chelating activity was calculated using the equation 1:

$$\% \text{ Chelating Activity} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}] \times 100$$

Statistical Analysis

Data were presented as means and standard deviations. All antioxidant assays were carried out in triplicate. A paired t-test was performed to determine the difference between the FCRRS content and the antioxidant capacity before and after simulated gastrointestinal digestion. The antioxidant capacity results were analyzed by ANOVA followed by the Tukey's test. The statistical analyses were carried out using the software SPSS 15.0 (SPSS Inc, Chicago, IL, USA).

Principal component analysis (PCA) was performed to understand how the phenolic compounds and the digestive process can influence the antioxidant behavior of the samples. Data were autoscaled and analyzed using Pirouette Statistics, version 3.11.

RESULTS AND DISCUSSION

Chemical characterisation of flaxseed products

The chemical composition (proteins, moisture, ash, lipids, and dietary fiber) of flaxseed products is shown in Table 1. The basis product of this study (FM) contained 29.3% protein, 8.4% moisture, 4.2% ash, 14.9% lipids, and 35.1% dietary fiber. Defatting FM resulted in a reduction of lipids content and a slight increase in both proteins and dietary fiber. DFM was subjected to ethanol treatment in order to remove polyphenols. This process slightly increased the protein content of phr-DFM (from 33.4 to 35.7%), as well as its moisture and fiber levels (Table 1). The resulting product, Phi, containing extracted polyphenols also contained a little amount of protein (5.21%), indicating that ethanol extraction also extracted some flaxseed protein from defatted meal. Protein extraction allow to obtain two products, FPC and phr-FPC which protein contents were 73.9 and 82.1%, respectively, 2.2- and 2.3-times higher than those obtained for their source products, DFM and phr-DFM. However, these values were lower than that reported in the literature.²⁷ This fact could be due to the extraction method employed that did not include seed coat removal previous to the defatting procedure. The levels of other components of FPC decreased after protein extraction, and they were similar to those reported in our previous study.¹⁶

FPC and phr-FPC were hydrolysed by Alcalase[®] for 180 min. The DH for both products was 18.3 ± 0.2 and $17.2 \pm 0.7\%$, respectively, with no significant differences between them ($p < 0.05$). These results suggest that the phenolic fraction present in protein concentrate did not interfere on Alcalase[®] activity.

Chromatographic analysis of flaxseed products: effects of enzymatic hydrolysis

The chromatographic profiles of flaxseed products before and after Alcalase[®] hydrolysis are shown in Figure 2. The chromatograms were obtained at 214 and 280 nm because these two wavelengths allow detecting both proteins/peptides and phenolic compounds.^{28,29} The 214 nm-chromatogram of FPC (Figure 2A) showed numerous peaks

eluting between 8 and 20 minutes which intensity was notably lower in phr-FPC and Phi products (Figure 2B and 2C). The intensity of a peak eluted at 15 min and detected at both 214 and 280 nm was much higher in the Phi than in FPC chromatogram, suggesting that it might correspond to a phenolic compound extracted with ethanol, as it was not observed in phr-FPC sample. Similarly, other components of lignan macromolecule present in FPC and Phi could elute as a wide peak with retention time between 20 and 23 min. This peak was also visible in the chromatographic profile of DFM product (data not shown). A previous analysis of flaxseed lignan macromolecule by size exclusion-HPLC with diode array detection, had also described the elution of a wide peak at 280 nm.¹⁴ The authors suggested that the heterogeneity of lignan macromolecule could be responsible for this result, considering the sample as a mixture of molecules with similar molecular weight but a slightly different composition of individual phenolic compounds. According to Johnsson, et al.³⁰, and Struijs, et al.³¹ SDG ester linked to hydroxymethyl-glutaric acid forms the backbone of the lignan macromolecule that is also comprised of the hydroxycinnamic acids, *p*-coumaric acid glucoside, and ferulic acid glucoside.

After hydrolysis with Alcalase[®], the chromatographic profiles of flaxseed products drastically changed in both appearance and intensity of eluted peaks. This was due to the release of peptides after the action of the microbial enzyme on flaxseed proteins. Profiles of FPH and phr-FPH (Figure 2D and 2E) were similar, indicating that phenolic compounds accompanying proteins in FPC did not affect Alcalase[®] activity, as it had been observed measuring the DH. Notable changes were also observed for Phh compared with Phi that could be due to modifications in phenolic compounds resulting from partial hydrolysis of lignan macromolecule under conditions (pH 8.5 and 60 °C) used to simulate enzymatic hydrolysis.

In order to identify phenolic compounds, FPH, phr-FPH, Phi, and Phh products were subjected to UPLC-MS/MS analysis, injecting standards under the same experimental

conditions. Figure 3A-3D shows the extracted ion chromatogram (EIC) of four phenolic compounds identified, ferulic, *p*-coumaric and caffeic acids, and SDG. Presence of these four compounds was confirmed in all samples except phr-FPH which is produced from a flaxseed product free of phenolic compounds. In the case of ferulic and *p*-coumaric acids (Figure 3A and 3B), more than one peak could be observed. Analysis of the mass spectra indicates that these peaks corresponded to modified versions of the phenolic compounds, mainly glycosylated forms, which fragment upon ionization conditions generated the same ions. These results strongly suggest that modified phenolic compounds are belonging to lignan macromolecule. According to Li, Yuan, Xu, Wang and Liu¹⁵ phenolic compounds in lignan macromolecule are firstly esterified by ethanol and subsequently hydrolysed by alkali to produce SDG and other phenolic glycosides. These phenolic glycosides and SDG were more clearly detected in Phh.

Protein-peptide-phenolic compounds complex

Once confirmed the presence of phenolic compounds in flaxseed products, potential interactions and the subsequent formation of complexes between proteins/peptides and polyphenols were investigated by fluorescence spectroscopy. Emission spectra from 290 to 500 nm of FPC, phr-FPC and Phi are shown in Figure 4A. Figure 4B shows spectra corresponding to FPH, phr-FPH and Phh samples.

The fluorescence of folded proteins is a result of the fluorescence from individual aromatic residues. Most of the intrinsic fluorescence emissions are due to excitation of Trp residues, with some emissions due to Tyr and Phe. Trp has an emission peak ranging from 308 to 350 nm depending on the local environment and the degree of solvent exposure of the chromophore.³² In our study, the highest fluorescence intensity was measured for phr-FPC and phr-FPH products which did not contain phenolic compounds. However, presence of

these components in FPC and FPH resulted in a notable reduction (~50%) of fluorescence intensity as well as a shift in the maximum emission peak (from 348 nm in FPC to 356 nm in phr-FPC, and from 358 nm in FPH to 360 nm in phr-FPH). This reduction could be due to modifications in protein/peptide chains caused by the formation of protein/peptide complexes with phenolic compounds in FPC and FPH products. Kanakis et al.²¹ had reported that when proteins interact with other molecules, Trp fluorescence changes depending on the impact of the interaction on the spatial conformation of the protein structure. A negligible emission was observed for Phi and Phh samples (Figure 4A and 4B), which chemical characterisation had demonstrated very low protein content.

Impact of simulated gastrointestinal digestion of peptide profile and antioxidant activity

The chromatograms of flaxseed products after simulated digestion, obtained at 214 and 280 nm, are shown in Figure 5 (A-F). As it can be observed, the action of digestive proteases on FPC and phr-FPC led to a high number of peptides that eluted between 8 and 20 min (Figure 5A and 5B). Both samples after digestion showed similar profiles, indicating that phenolic compounds present in FPC did not affect the enzymatic action of pepsin and pancreatin. Only the wide peak eluting at 20-23 min, corresponding to lignan macromolecule, was still visible in the chromatogram of digested FPC (dFPC). The same behavior was observed for FPH and phr-FPH, samples resulting from Alcalase[®] hydrolysis (Figures 5D and 5E). Moreover, these profiles were similar to those shown by their digested parent products (dFPC and dphr-FPC), suggesting that peptides visible in the chromatograms were released by the action of pepsin and pancreatin on flaxseed proteins that had been not previously degraded by the microbial enzyme.

In the case of phenolic compounds fraction (Phi and Phh), different behavior was observed after their simulated digestion. Small differences in the profile of digested Phi (dPhi,

Figure 5C) were detected comparing with non-digested sample (Phi, Figure 2C) that could be due to phenolics modifications caused by pH and temperature changes. Analysis of Phh and digested Phh (dPhh) showed similar chromatographic patterns (Figure 2F and 5F), thus indicating that phenolic compounds modified by alkaline conditions were not further affected by digestive conditions.

In order to evaluate the potential contribution of protein/peptide and phenolic compounds on the antioxidant capacity of flaxseed, different products were subjected to analysis using several measuring methods (ORAC, FRAP, FCRRS, and metal chelating activity). The results would also allow evaluating the impact of Alcalase[®] hydrolysis and gastrointestinal digestion on the antioxidant activity. Figure 6 shows the results obtained from different assays before (Figure 6A, 6C, 6E, and 6G) and after simulated digestive process (Figure 6B, 6D, and 6F).

Before simulated digestion, Phi and Phh products showed the highest ORAC and FRAP values as well as the greatest chelating potential. This might be associated with the higher concentration of nucleophilic centers comparing with other flaxseed products, as well as with the possible synergisms among different phenolic compounds. The ability of the phenolic compounds to act as multifunctional antioxidant, as chain-breaking or metal chelating agent, can be explained by the nucleophilic character of the aromatic rings in its structure.³³ The ORAC value of Phh was 1.5-times higher than that of Phi, which can be related to the release of compounds of lower molecular weight and lower hydrophobicity resulting from alkaline conditions, as it was observed by chromatographic analysis (Figure 2C, 2F). Among these compounds, glycosylated ferulic, caffeic, and *p*-coumaric acids could be responsible for the increase in the ORAC value. These phenolic acids act as antioxidant mainly through a hydrogen atom (H^+) transfer mechanism due to the reactivity of their phenol moiety, although they also could act via electron donation.³⁴ The iron chelating ability of Phi

and Phh was similar ($p > 0.05$) (Figure 6G), and about 2-times higher than that of FPH and phr-FPH. The potential of FPH and phr-FPH may be compromised by the dietary fiber content (Table 1) because these high molecular weight polysaccharides could interfere with the iron-peptides interaction, hindering the formation of the chelate.³⁵ No significant differences were observed between both hydrolysates, suggesting that their potential is due to peptides, especially those containing His, Glu, Asp, and Cys residues³⁶ rather to the presence of polyhydroxylated rings in phenolic compounds. Some studies have shown that iron chelating by peptides may facilitate absorption of this mineral by intestinal cells³⁷⁻³⁹ increasing its bioavailability, while some classes of phenolic compounds may exert an opposite effect.⁴⁰ However, it has been demonstrated that iron chelating by peptides or phenolic compounds may maintain the metal more stable and less prone to interactions,⁴¹ which prevents free iron to catalyze human body reactions involving ROS, leading to the oxidation of unsaturated lipids and promoting oxidative damage in cells.⁴² Thus, both peptides as phenolic compounds can have a beneficial effect due to its ability to chelate iron.

Alcalase[®] hydrolysis was responsible for an increase in the antioxidant potential of flaxseed products up to 6 and 4 times, as determined by ORAC and FRAP assays, respectively, compared with non-hydrolysed products. Similar results have been previously reported for other plant protein hydrolysates.⁴³ Although the absolute values of the antioxidant capacity of samples containing protein and phenolic compounds were higher than those measured for products only containing proteins, the relative increase on the antioxidant capacity as a result of hydrolysis with Alcalase[®] was similar. Therefore, this increase might be associated with the release of peptides during enzymatic hydrolysis rather than with changes in phenolic compounds.¹ FPH showed the highest FCRRS content ($p < 0.05$) with a value 2- and 1.4-times higher than that measured in phr-FPH and Phi products, respectively (Figure 6E), indicating that exposure of both aromatic rings of the phenolic compounds and

aromatic residues of proteins during hydrolysis with Alcalase[®] was responsible for this antioxidant mechanism of action.

As expected, the antioxidant capacity of flaxseed products was maintained or increased after gastrointestinal digestion (Figure 6B, 6D, and 6F), indicating that the digestive process might exert a beneficial effect on the release of bioactive compounds, regardless of the mechanism evaluated. This effect was higher than that demonstrated for Alcalase[®] hydrolysis. In the case of the effect of digestion on the antioxidant capacity of Phi, a significant increase of FRAP value and FCRRS content was observed, while ORAC value did not change after the action of digestive enzymes. However, three antioxidant values were increased when simulated digestion was performed on Phh, indicating that alkaline conditions favored the access of nucleophilic sites of phenolic compounds to radicals in spite of chromatographic profiles of Phh and dPhh were similar. In literature, the effect of digestion on the phenolic compounds antioxidant capacity is contradictory and dependent on the digested product. Tarko et al.⁴⁴ showed that the antioxidant capacity of the phenolic compounds from apple and plum increased, while those from pear and banana decreased after simulated digestion. The antioxidant activity of FPC and phr-FPC, without previous Alcalase[®] hydrolysis, significantly increased after being subjected to simulated gastrointestinal digestion. Although the chromatographic profile of dFPC and dphr-FPC were similar, the antioxidant behavior was different, with highest capacity shown by product containing both proteins and phenolic compounds. This was also observed for products previously hydrolysed by Alcalase[®] (dFPH and dphr-FPH).

Influence of phenolic compounds on antioxidant behavior and antioxidant capacity of flaxseed products

PCA was performed to understand how the presence of phenolic compounds influenced the antioxidant potential of flaxseed products. PCA displays similarities and differences among samples from their spatial distribution. ORAC, FRAP and FCRRS values were considered as independent variables, while flaxseed deffated flours, protein concentrates and hydrolysates, and their digests were the dependent variables. Samples were then distributed according to different oxidation assays, allowing knowing the predominant antioxidant mechanism for each sample (Figure 7).

Samples were centered on two main axes or principal components (PC), PC1 that explained 89.3% of the variance and PC2 that explained 9.7%. Thus, both components explained 99.0% of the antioxidant behavior of flaxseed products. As it can be observed in Figure 7, FPC and phr-FPC are located in the same quadrant, indicating that there are no differences between the antioxidant mechanisms of these samples. Same results were obtained for source flours, DFM and phr-DFM. However, once subjected to Alcalase[®] hydrolysis, the behavior of the hydrolysates changed, and FPH and phr-FPH were located in opposite quadrants. Phr-FPH, only containing peptides, was located at the downleft quadrant, indicating that its antioxidant activity was mainly mediated through protons transference and peroxy radicals chelation. However, FPH, containing both peptides and phenolic compounds, was located at the upper right quadrant, suggesting that phenolic compounds were the main responsible for reducing ferric to ferrous iron (FRAP assay). After simulated gastrointestinal digestion, similar behavior was observed with peptides released from the action of digestive enzymes responsible for ORAC and FCRRS values, and phenolic compounds contributing to iron reduction.

The presence of polyphenols contributed positively, but in a variable way, on the antioxidant capacity of the majority of protein samples. Comparing FPC and phr-FPC, the presence of phenolic compounds led to an increase of about 80% on antioxidant capacity measured by ORAC and the FRAP assays. For the hydrolysate, the presence of phenolic compounds also led to an increase of nearly 80%, measured by FRAP, but only 15% as measured by ORAC. After simulated digestion, the influence of the phenolic compounds on the antioxidant capacity of the samples determined by the FRAP remained high, between 70 and 80%, while lower influence was observed when the antioxidant capacity was measured by ORAC (Figure 6). This impact can be explained either by synergism between the antioxidant compounds or formation of protein-phenolic complex. In proteinaceous samples, antioxidants compounds are SDG, caffeic, ferulic and p-coumaric acids,³⁴ as well as flaxseed peptides, in Alcalase[®] hydrolysates or in digested samples.⁴⁵ Together, such compounds may have their antioxidant potential increased, since a non-oxidized compound is able to regenerate the other which has been oxidized, in a similar way that synergism occurs between α -tocopherol and flavonoids or α -tocopherol and ascorbate.⁴⁶

In the protein concentrates, the positive influence of phenolic compounds on antioxidant capacity can be either due to their high amount of phenolic hydroxyl groups and/or by unfolding the protein structure due to protein-phenolic complex formation. In most cases, formation of complexes increases the exposure of nucleophilic centers formed by hydrophobic amino acid residues at the N-terminal portion, or the presence of His, Trp, Phe, Tyr, Cys in the protein moieties.¹ Although the formation of complex promotes the participation of protein as an antioxidant, it can, in turn, compromise the performance of polyphenols in this process, masking their bioactivity.⁴⁷ Thus the resulting antioxidant activity is due the increasing the antioxidant capacity of proteins and decreasing that of phenolic compounds.

On the other hand, in the hydrolysates, the formation of complex is hindered due to the small contact surface between peptides and phenolic compounds.⁴⁸ Thus protein hydrolysis can weaken the protein-phenolic complexes, by reducing the interactions between these compounds and increasing both the availability of the nucleophilic sites of peptides and phenolic compounds. This greater exposure of regions capable of neutralizing reactive species and the possible synergism between these two classes of compounds may also explain the higher antioxidant capacity of the hydrolysates when compared to the concentrates.

In conclusion, the phenolic fraction showed the highest antioxidant capacity among the flaxseed products studied, which was enhanced by both alkaline hydrolysis and simulated gastrointestinal digestion, possibly by releasing SDG and *p*-coumaric, caffeic and ferulic phenolic acids. The hydrolysis by both Alcalase[®] and digestive enzymes also resulted in an increase of the antioxidant activity of protein concentrates with/without phenolic compounds. Peptides released act through protons transference and peroxy radicals chelation while phenolic compounds were, furthermore, responsible for the iron reduction.

The formation of protein-phenolic complexes may have a positive effect on the antioxidant capacity of plant protein concentrates. In our study, we have found that flaxseed protein-phenolic complexes favored the exposure of protein moieties capable of acting as an antioxidant, which would complement the antioxidant potential of phenolics, with a positive relationship between these two classes of compounds. This would have a dual effect of phenolic protection against oxidative degradation along the gastrointestinal tract and establishment of a positive antioxidant environment. Animal models should be needed to evaluate the bioavailability of peptides and phenolic compounds as well as to confirm the *in vivo* antioxidant effects providing health benefits against oxidative stress-associated disorders.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGMENTS

The authors thank FAPESP (2010/52680-7) for the financial support, CNPq and UNICAMP (FAEPEX 607/11) for the scholarship granted to F.G.D. Silva, and the Laboratory of Spectroscopy and Calorimetry (Brazilian Biosciences National Laboratory) for the support with fluorescence analysis. We also want to thank Professor Márcia Miguel Castro Ferreira for the support with Principal Component Analysis and Waters Brazil (São Paulo, SP, Brazil) for supplying the UPLC-Xevo G2-XS QToF system employed in this study. B.H-L. thanks CSIC and the Ministry of Economy and Competitiveness for her and Ramón y Cajal contract.

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FIGURE CAPTIONS

Figure 1. Flow chart of the preparation of flaxseed products from flaxseed meal (FM)

Figure 2. Chromatogram profiles (RP-HPLC) recorded at 214 nm and 280 nm of flaxseed products before and after Alcalase[®] hydrolysis. (A) flaxseed protein isolate (FPC), (B) phenolic reduced flaxseed protein isolate (phr-FPC), (C) phenolic compounds isolate (Phi), (D) flaxseed protein hydrolysate (FPH), (E) reduced phenolic flaxseed protein hydrolysate (phr-FPH), and (F) phenolic compounds hydrolysate (Phh)

Figure 3. Extracted ion chromatogram (EIC) of (A) ferulic acid (m/z 193.1 \pm 0.5), (B) p-coumaric acid (m/z 163.0 \pm 0.5), (C) caffeic acid (m/z 179.0 \pm 0.5) and (D) secoisolariciresinol diglucoside (SDG) (m/z 685.3 \pm 0.5). EICs correspond (from bottom to top) to phenolic reduced flaxseed protein (phr-FPH), flaxseed protein hydrolysate (FPH), phenolic compounds hydrolysate (Phh) and phenolic compounds isolate (Phi)

Figure 4. Fluorescence emission spectra of (A) flaxseed protein isolate (FPC), phenolic reduced flaxseed protein isolate (phr-FPC) and phenolic compounds isolate (Phi), and (B) flaxseed protein hydrolysate (FPH), phenolic reduced flaxseed protein (phr-FPH) and phenolic compounds hydrolysate (Phh).

Figure 5. Chromatogram profiles (RP-HPLC) recorded at 214 nm and 280 nm of flaxseed products after simulated gastrointestinal digestion. (A) Digested flaxseed protein isolate (dFPC), (B) Digested phenolic reduced flaxseed protein isolate (dphr-FPC), (C) digested phenolic compounds isolate (dPhi), (D) digested flaxseed protein hydrolysate (dFPH), (E) digested reduced phenolic flaxseed protein hydrolysate (dphr-FPH), and (F) digested phenolic compounds hydrolysate (dPhh)

Figure 6. Antioxidant capacity of flaxseed protein products determined by ORAC (A and B), FRAP (C and D), FCRRS levels (E and F) and quelating capacity (G), before (A, C, E, G) and after simulated gastrointestinal digestion (B, D, F). Values expressed as mean of duplicates

635 (each in triplicate) \pm standard deviation. Bars with different letters differ from each other by
636 ANOVA, post hoc Tukey test ($p < 0.05$). Bars of the same sample with phenolics and reduced
637 phenolics with ‘*’ are different from each other by T test ($p < 0.05$).

638 **Figure 7.** Principal component analysis (PCA) on the measured parameters: ORAC, FRAP
639 and FCRRS before and after Alcalase hydrolysis and simulated gastrointestinal digestion. (▼)
640 Defatted flaxseed meal (DFM); (◆) Phenolic reduced defatted flaxseed meal (phr-DFM); (○)
641 Flaxseed protein isolate (FPC); (□) Phenolic reduced flaxseed protein isolate (phr-FPC); (Δ)
642 Flaxseed protein hydrolysate (FPH); (■) Phenolic reduced flaxseed protein hydrolysate (phr-
643 FPH); (◇) Digested DFM (dDFM); (*) Digested phr-DFM (dphr-DFM); (♣) Digested FPC
644 (dFPC); (♥) Digested phr-FPC (dphr-FPC); (+) Digested FPH (dFPH); (♠) Digested phr-FPH
645 (dphr-FPH).

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TABLES

Table 1. Chemical composition (expressed as %) of flaxseed meal (FM), defatted flaxseed meal (DFM), phenolic reduced defatted flaxseed meal (phr-DFM), flaxseed protein isolate (FPC), phenolic reduced flaxseed protein isolate (phr-FPC), flaxseed protein hydrolysate (FPH), phenolic reduced flaxseed protein hydrolysate (phr-FPH), phenolic isolate (Phi) and phenolic hydrolysate (Phh). Results are the mean \pm standard deviation

Sample	Proteins ^a	Moisture	Ash	Lipids	Dietary Fiber
FM	29.28 \pm 0.38	8.42 \pm 0.00	4.20 \pm 0.00	14.86 \pm 0.87	35.12 \pm 0.31
DFM	33.36 \pm 0.94	8.18 \pm 0.16	4.48 \pm 0.03	6.83 \pm 0.17	38.84 \pm 0.32
phr-DFM	35.68 \pm 0.01	11.94 \pm 0.31	4.44 \pm 0.04	6.41 \pm 0.06	40.73 \pm 0.49
FPC	73.91 \pm 1.00	3.82 \pm 0.12	1.57 \pm 0.05	4.73 \pm 0.17	13.01 \pm 0.29
phr-FPC	82.05 \pm 1.04	3.07 \pm 0.22	1.64 \pm 0.08	1.11 \pm 0.05	14.91 \pm 0.11
FPH	67.85 \pm 0.22	ND	ND	ND	11.94 \pm 0.27
phr-FPH	75.18 \pm 0.31	ND	ND	ND	13.67 \pm 0.08
Phi	5.21 \pm 0.17	ND	ND	ND	ND
Phh	4.77 \pm 0.13	ND	ND	ND	ND

^a N (%) \times 6.25; ND: Not determined